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A New Fractionation Method for Humic Acids

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Abstract. It has been shown that the two-phase systems introduced by Albertsson can be used for fractionation of humic acids. Counter-current distribution in our aqueous dextran-polyethylene glycol system demonstrated heterogeneity of humic acid. There are complications, which disturb the counter-current fractionation.

INTRODUCTION

Humic acids are very heterogenous substances, so heterogenous that it has even been suggested that it does not make sense to try to isolate well-defined compounds. Even those who take that pessimistic attitude probably agree that great efforts should be made to obtain as well-defined fractions as possible. Unfortunately most separation methods known from organic chemistry and biochemistry have been applied with rather meager and uncertain results. The most successful application so far seems to be molecular sieving with Sephadex or glassbeads where the fractionation probably takes place according to molecular volume, even if this has never been proven with independent methods. Any use of electrophoresis on paper and other supporting substances has been strongly disturbed by adsorption effects. Free electrophoresis would, however, in principle give a separation by negative charge and should be tried carefully. Albertsson has developed a separation method in aqueous two-phase systems which has shown great potential for separations in biological systems (Albertsson, 1971). This method has now been used for fractionation of humic acids and the results are presented in the following paper.

EXPERIMENTAL

The substance referred to as humic acid or humic acid 1 is extracted at pH 7 with sodium pyrophosphate as described by Lindqvist (1982).

In one experiment another humic acid, humic acid 2, from a *chernozem* type of soil has been used.

Four different fractions from humic acid have also been prepared according to Lindqvist (1968). They are marked N1, N2, N4 and N6.

To prepare the two-phase systems polyethylene glycol, PEG-6000 (melting point 60–63°C), and Dextran T 70 and T 500 (Pharmacia) are used. They are all dissolved in buffers; 0.1 M citrate-HCl (pH 3), 0.1 M acetate (pH 4 or 5), 0.1 M citrate (pH 6) or 0.05 M Tris-HCl (pH 7, 8 or 9). In the two-phase systems the concentrations are: 8% Dextran T 70

Table 1. *Absorbance ratios top to bottom for humic acids in two-phase systems*

	Wave length (nm)		
	400	500	600
Humic acid 1, pH=5 in			
PEG-6000 + Dextran T 70	–	1.34	–
PEG-6000 + Dextran T 500	–	1.66	–
PEG-6000 + Dextran T 500, pH=3 with			
humic acid 1	3.82	–	4.37
humic acid 2	1.78	–	1.70
PEG-6000 + Dextran T 70, pH=7 with			
humic acid 1	0.88	–	0.72
humic acid 2	0.21	–	0.18

Table 2. *Absorbance ratios top to bottom at 500 nm for humic acids fractionated in a two-phase system of PEG-6000 and Dextran T 500*

	pH			
	3	5	7	9
N1	3.5	1.2	0.8	0.9
N2	5.5	1.4	1.2	1.0
N4	3.5	1.2	1.4	1.2
N6	4.0	1.3	1.0	1.0
Positively charged PEG				
N1	6.2	3.4	2.6	4.2
N2	10.4	6.8	3.5	6.2
N4	5.1	5.8	2.6	8.8
N6	5.7	7.2	5.2	9.0

Table 3. β'- *and k-values for a number of CCD fractionations. All values are based on* $A_{400nm} \geqslant 0.05$.

	Tube number								
	0	1	2	3	4	5	6	7	8
Humic acid 2, 0.1% at pH=3									
Top phase k	–	–	3.4	3.9	4.8	5.5	4.5	5.3	5.2
Top phase A_{400}	0.048	0.098	0.165	0.242	0.314	0.384	0.453	0.465	0.488
β'	0.12	0.21	0.28	0.38	0.46	0.55	0.68	0.81	1.09
Bottom phase A_{400}	0.398	0.471	0.580	0.641	0.684	0.704	0.662	0.572	0.447
Bottom phase k	2.4	3.1	3.2	3.3	3.8	4.0	4.0	4.3	4.6
Humic acid 1, 0.1% at pH=3									
Top phase k	–	–	–	–	–	–	–	–	8.2
Top phase A_{400}	–	–	–	–	0.050	0.101	0.176	0.286	0.411
β'	–	–	–	–	0.86	1.06	1.24	1.52	1.93
Bottom phase A_{400}	–	–	–	0.052	0.058	0.095	0.142	0.188	0.213
Bottom phase k	–	–	–	–	–	–	–	–	–
Humic acid 1, 0.1% at pH=9									
Top phase k	–	–	–	5.4	6.2	6.7	7.3	7.5	8.6
Top phase A_{400}	–	0.075	0.137	0.264	0.456	0.679	0.866	0.927	0.866
β'	–	0.25	0.32	0.43	0.51	0.62	0.70	0.79	0.92
Bottom phase A_{400}	0.219	0.306	0.429	0.609	0.897	1.097	1.234	1.167	0.941
Bottom phase k	3.4	3.1	4.3	5.3	5.1	6.1	6.8	6.9	7.7
Humic acid 1, 0.2% at pH=7									
Top phase k	–	–	–	6.6	7.2	7.4	7.6	7.7	7.7
Top phase A_{400}	–	0.047	0.143	0.354	0.751	1.228	1.709	2.062	2.088
β'	–	0.45	0.55	0.62	0.67	0.72	0.77	0.83	0.90
Bottom phase A_{400}	0.061	0.104	0.258	0.573	1.118	1.714	2.218	2.498	2.316
Bottom phase k	–	–	4.6	6.0	6.5	6.4	7.0	7.4	6.8
Reduced humic acid 1, 0.2% at pH=7									
Top phase k	–	–	4.0	4.5	5.9	6.4	6.5	6.8	6.3
Top phase A_{400}	0.044	0.100	0.198	0.440	0.730	1.145	1.437	1.647	1.527
β'	(0.054)	(0.60)	0.51	0.55	0.59	0.67	0.75	0.88	1.05
Bottom phase A_{400}	0.082	0.167	0.387	0.800	1.239	1.718	1.926	1.865	1.599
Bottom phase k	1.8	3.0	3.5	4.8	5.2	5.8	5.6	5.9	6.1
Fraction N1, 0.2% at pH=7									
Top phase k	–	1.5	3.8	6.2	7.7	7.7	8.3	7.5	7.8
Top phase A_{400}	0.057	0.150	0.180	0.486	0.881	1.440	1.902	1.797	1.818
β'	(0.90)	(1.38)	(0.70)	0.68	0.71	0.76	0.79	0.90	0.93
Bottom phase A_{400}	0.063	0.109	0.257	0.718	1.241	1.900	2.408	1.987	1.954
Bottom phase k	–	2.1	4.0	6.0	6.5	7.8	7.9	7.4	8.5
Fraction N2, 0.2% at pH=7									
Top phase k	–	–	2.7	4.8	5.4	5.8	5.6	5.9	6.1
Top phase A_{400}	–	0.063	0.147	0.304	0.576	0.910	1.217	1.289	1.186
β'	–	(0.54)	0.53	0.59	0.67	0.76	0.86	0.93	1.09
Bottom phase A_{400}	0.055	0.117	0.278	0.514	0.863	1.199	1.409	1.386	1.089
Bottom phase k	–	–	3.4	4.1	4.8	5.1	5.5	5.7	6.1
Fraction N2, 0.2% at pH=7, positively charged PEG									
Top phase k	–	–	–	–	–	6.6	7.3	6.5	6.6
Top phase A_{400}	–	–	–	0.118	0.236	0.370	0.575	0.865	1.124
β'	–	–	–	(0.31)	0.93	1.08	1.17	1.37	1.56
Bottom phase A_{400}	0.079	–	–	0.090	0.254	0.344	0.492	0.630	0.721
Bottom phase k	0.17	–	–	–	–	4.6	6.0	6.1	6.1
Theoretical β-values									
	0	0.07	0.15	0.25	0.36	0.50	0.67	0.88	1.14

9	10	11	12	13	14	15
5.1	5.0	4.9	4.7	4.6	4.6	4.4
0.499	0.518	0.548	0.544	0.478	0.411	0.180
1.37	1.79	2.28	3.24	3.59	3.64	(2.14)
0.365	0.290	0.240	0.168	0.133	0.113	0.084
4.4	3.6	4.4	3.0	2.8	2.5	–
8.3	8.0	7.4	7.5	7.2	7.0	7.2
0.512	0.717	0.905	1.119	1.287	1.246	0.886
2.28	3.08	3.85	6.32	10.1	16.8	18.5
0.225	0.233	0.235	0.177	0.127	0.074	0.048
–	–	5.3	–	–	–	–
8.5	8.4	8.0	–	–	–	–
0.721	0.563	0.409	0.276	0.166	0.082	–
1.08	1.36	1.65	1.99	2.24	(1.61)	–
0.666	0.413	0.248	0.139	0.074	0.051	–
6.2	7.2	5.8	–	–	–	–
7.8	7.7	6.6	6.6	–	–	–
1.778	1.333	0.802	0.408	0.193	0.074	–
1.01	1.17	1.38	1.50	2.00	(1.45)	–
1.762	1.139	0.585	0.271	0.096	0.051	–
6.3	5.6	4.6	3.6	–	–	–
6.0	6.5	6.1	5.3	3.7	–	–
1.362	0.954	0.571	0.312	0.157	0.078	–
1.05	1.21	1.43	1.53	1.69	(1.18)	–
1.248	0.787	0.398	0.204	0.093	0.066	–
6.1	5.4	5.9	4.6	–	–	–
7.8	6.6	4.9	4.7	2.7	–	–
1.364	0.793	0.472	0.234	0.125	0.110	0.171
1.00	1.09	1.20	1.61	1.81	(1.07)	(0.83)
1.360	0.727	0.392	0.145	0.069	0.103	0.085
8.5	7.3	5.0	–	–	2.0	–
5.7	5.3	4.9	4.7	4.1	4.0	3.7
0.951	0.738	0.611	0.465	0.351	0.313	0.177
1.30	1.63	2.02	2.24	3.86	5.05	2.53
0.734	0.454	0.302	0.208	0.091	0.062	0.070
6.0	5.0	5.0	3.5	–	–	–
6.2	5.7	5.5	5.5	5.3	5.3	5.1
1.390	1.562	1.640	1.684	1.572	1.163	0.540
1.95	2.13	3.84	5.43	8.15	9.23	(7.40)
0.713	0.586	0.427	0.310	0.193	0.126	0.073
6.0	5.4	4.5	5.3	–	–	–
1.50	2.00	2.75	4.50	6.50	14.0	∞

or T 500, 5.76% PEG-6000 and 0.02% or 0.01% humic acid.

The normal procedure is to have stock solutions of higher concentrations and then weigh out Dextran, PEG and pH-buffer in test tubes. The humic acid is dissolved in the buffer.

For the counter current distribution experiments, a sufficient amount of Dextran, PEG and buffer are mixed and 5 g portions are withdrawn from the solution and put in test tubes. It is important to stir the solution. The test tubes are left to form the two phase system. The volume ratio is approximately 1. In the starting test tube, the concentration of humic acid is 0.1% or 0.2%.

The effect of positively charged PEG is studied in the same way with 5% of the PEG-6000 changed to hexaetonium-PEG. The hexaetonium-PEG has been made by and given to us by Dr Göte Johansson, Department of Biochemistry, Box 740, S-220 07 Lund, Sweden.

RESULTS AND DISCUSSIONS

The humic acids are distributed between the two phases

The separation method would be of no value if the humic acids always were collected in either the top or the bottom phase or if they all had the same distribution ratio. Albertsson (1971) has indicated many possibilities for shifting the distribution ratios in the desired direction and it was easy to find two-phase systems which directly gave distribution ratios near one.

The distribution ratios were never determined analytically. The absorbance ratios of the top to the bottom phase, β', at different wave lengths have been used and the fact that these ratios vary with the wave length (Table 1) proves that the fractions in the top and bottom phase are not identical. From Table 1 one can infer that the absorbance ratio, top-to-bottom, increases with increasing molecular weight of the dextran used, that different humic acids give different absorbance ratios and that the absorbance ratios vary very much with pH (*vide infra*). The possibilities for separation should thus be good.

Effects of pH variation

Table 2 shows the absorbance ratios for four different humic acid fractions of the same soil fractionated at four different pH's. The most obvious observation, also confirmed in Table 1, is the high ratios at the lowest pH. No explanation can at present be given of this trend which might be a property of the two-phase system or of the humic acid. It has been pointed out by Albertsson (1971) that a proper comparison of pH variations can only be made in the presence of an excess of a defined neutral salt. The experiments reported here are only aimed at showing the possibilities for varying the separation conditions.

It is also shown in the table that the introduction of a positively charged PEG in the two-phase system, dominating the top phase will increase the β'-values at all pH's.

Counter-current distributions

For practical separations Albertsson recommends use of counter-current distribution methods (Albertsson, 1971). Before the practical applications can be made it is, however, important to study what actually happens in such counter current experiments with a humic acid.

All work reported here has been done without special CCD-apparatus which makes the distribution values reported safer but prevents the use of as many steps as in the practical applications. More accurate work in a limited number of steps (here 15) should however give clear ideas about the applicability of the method. The width of any component will be appreciable with the small number of steps used. For a theoretical (*vide infra*) β-value of 1.00 the resulting distribution would have its maximum between steps 7 and 8 and half the maximum values would still prevail at steps 5 and 10. The overlap will thus be very large but the homogeneity can be judged from the variation of the β-values (here the experimental β'-values) with the number of step. For *one* component the β-values would be constant, independent of the steps and would agree with the theoretical b-values at the maxima. (The theoretical β-values are calculated from the formula $\beta=r/(n-r)$. The number of tube is r and the total number of steps is n). The results of a number of selected experiments are represented in Table 3.

It is easy to see that the measured β-values are neither constant in any appreciable range of steps nor agree with the theoretical values except in a few instances. *The heterogeneity of the samples is thus obviously large.*

Before we proceed any further some anomalies must be pointed out. A number of β'-values are set within brackets. They do not follow the expected order from smaller values in the earlier steps to larger values at the end of the CCD trail. One could imagine different reasons for these anomalies: (1) It has been observed that the dextran phase is saturated with humic acid already at a 0.01% in the bottom phase, (2) There is a slight adsorption of humic acid to the dextran phase as earlier observed for Sephadex (Lindqvist, 1967), giving rise to too small β-values at low concentrations, (3) The different fractions have appreciably different absorption spectra so that β' is not a satisfactory measure of the concentration distribution. It is difficult to repeat the counter-current experiments in a perfectly reproducible way, at least manually. The trends reported in Table 3 are, however, found in all repetitive experiments. A separation is thus achieved which varies slightly from one experiment to another.

Taking all these reservations into consideration and using only $A \geqslant 0.05$ some rather definite observations can still be made from the results reported in Table 3:

1. The k values ($k=A_{400}/A_{600}$), which are characterizations of humic acids, vary in a regular way with smaller values at the beginning and end of the CCD-trail. In Sephadex and glass bead molecular sieving a different variation of k has been observed (Lindqvist & Lindqvist, 1979). The fractionation principle can therefore not be the same in the two methods. The two-phase method thus fractionates the humic acid in a new way with a variation of the β'-values up to a factor or 10. It remains to find out what governs the fractionation. The pH-variations reported in Tables 1 and 2 indicate that the negative charge is the dominating factor, if negatively charged ions prefer the bottom phase. The introduction of positively charged PEG, dominating in the top phase does increase the β'-values as expected. It is, however, surprising that this increase is very large also at low pH where the negative charge of the humic acids should be relatively small.

The comparison between humic acid 1 and 2 shows that the β-values are in general much lower for humic acid 2. This humic acid is certainly more aromatic than humic acid 1 which indicates that the aromatic character influences the distribution between the two phases. The problem will be studied further.

2. The range of β'-values observed is a clear indication of a considerable heterogenity. The cases of special interest are where the k-values of the top phase differ appreciably from those of the bottom phase. The most obvious example is humic acid 2 where the maxima in the absorbance values differ much between the top and bottom phase as well as the k-values.

3. The differences between humic acids from two different soils is much larger than between different fractions of the humic acid from one soil. The different fraction in the latter case seem to represent only minor variations in the composition of the same heterogeneous material. The reduced humic acid (Lindqvist, 1983) is also very similar to the unreduced one.

4. The two-phase fractionation method should, combined with other fractionation methods, lead to an increased fractionation of humic acids.

5. For this to be achieved it is, however, essential to find fast and good methods to separate the humic acids from the two-phase system so that they can be concentrated before repetition of the fractionation or before starting other types of fractionation procedures. One possibility is to precipitate the humic acid and dissolve it again to a higher concentration. This is unfortunately a very time-consuming procedure which is also hampered by a protective effect of the PEG against precipitation. Other methods are now being studied.

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